

Maternal Ezetimibe Concentrations Measured in Breast Milk and its Use in Breastfeeding Infant Exposure Predictions

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Running heading: Ezetimibe Measured in Breast Milk and Infant Exposure Predictions

DECLARATIONS

Funding: This research was funded by the Canadian Institutes of Health Research (CIHR) Project Grant, PJT-159782; and CIHR Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award (CGS-D), a Canada Scholarship to Honour Nelson Mandela, DF2-171445.

Conflicts of interest: The authors have no conflicts of interest relevant to this article to disclose.

Availability of data and material: A de-identified dataset can be shared with the appropriate approval. The requestor would need to contact the PI (Dr. Shinya Ito, shinya.ito@sickkids.ca) with the request.

Ethics approval: This study received ethics clearance from the Research Ethics Boards of the University of Manitoba (#HS19991) and the University of Waterloo (REB # 41155).

Informed consent statement: Informed consent was obtained from all individual participants included in the study.

Code availability: Not applicable.

Author contributions: C.H.T.Y., S.I., and A.N.E. conceptualized and designed the study. J.A., A.D., and Y.T. developed the assay. P.D., P.K., and M.R. recruited patients and performed data collection. C.H.T.Y. wrote the original draft manuscript. All authors reviewed and revised the manuscript.

47
48 **ACKNOWLEDGEMENTS**

49 We would like to acknowledge Medela Canada Inc. for providing electric breast pumps for this
50 study. We would also like to acknowledge the Lipid Clinic at St. Boniface Hospital for their
51 contributions in arranging the collection of breast milk samples and storage, and completing
52 patient questionnaires.

53 This work is based on Cindy Hoi Ting Yeung's thesis presented to the University of Waterloo in
54 partial fulfilment of the requirement for the degree of Doctor of Philosophy in Pharmacy and a
55 copy of the thesis is available from the University of Waterloo website at:

56 [https://libuwspaceprd02.uwaterloo.ca/bitstream/handle/10012/19412/Yeung_CindyHoiTing.pdf?i](https://libuwspaceprd02.uwaterloo.ca/bitstream/handle/10012/19412/Yeung_CindyHoiTing.pdf?isAllowed=y&sequence=7)
57 [sAllowed=y&sequence=7](https://libuwspaceprd02.uwaterloo.ca/bitstream/handle/10012/19412/Yeung_CindyHoiTing.pdf?isAllowed=y&sequence=7).

ABSTRACT

Background: Lactating mothers taking ezetimibe, an anti-hyperlipidemic agent, may be hesitant to breastfeed despite the known benefit of breastfeeding to both mother and infant. Currently, no data exists on the presence nor concentration of ezetimibe and its main active metabolite, ezetimibe-glucuronide (EZE-glucuronide), in human breast milk.

Methods: Voluntary breast milk samples containing ezetimibe and EZE-glucuronide were attained from lactating mothers taking ezetimibe as part of their treatment. An assay was developed and validated to measure ezetimibe and EZE-glucuronide concentrations in breast milk. A workflow that utilized a developed and evaluated pediatric physiologically based pharmacokinetic (PBPK) model, the measured concentrations in milk, and weight-normalized breast milk intake volumes was applied to predict infant exposures and determine the upper area under the curve ratio (UAR).

Results: Fifteen breast milk samples from two maternal-infant pairs were collected. The developed LCMS/MS assay showed an analytical range of 0.039-5.0 ng/mL and 0.39-50.0 ng/mL for ezetimibe and EZE-glucuronide, respectively. The measured concentrations in the breast milk samples were 0.17-1.02 ng/mL and 0.42-2.65 ng/mL of ezetimibe and EZE-glucuronide, respectively. The evaluated pediatric PBPK model demonstrated minimal exposure overlap in adult therapeutic dose and breastfed infant simulated AUC_{0-24h} . Calculated UAR across infant age groups ranged from 0.0015-0.0026.

Conclusions: PBPK model-predicted ezetimibe and EZE-glucuronide exposures and UAR suggest that breastfeeding infants would receive non-therapeutic exposures. Future work should involve a “mother-infant pair study” to ascertain breastfed infant plasma ezetimibe and EZE-glucuronide concentrations to confirm the findings of this work.

81 **KEY POINTS**

- 82 • An LCMS/MS assay was developed to measure ezetimibe and ezetimibe-glucuronide
83 (EZE-glucuronide) levels in voluntary breast milk samples and showed that
84 concentrations were low (0.17-2.65 ng/mL).
- 85 • Infant physiologically-based pharmacokinetic models using the measured ezetimibe and
86 EZE-glucuronide concentrations in milk for dose estimation predicted breastfeeding
87 infant exposures to be low.
- 88 • Quantifying breastfeeding infant exposure to maternal ezetimibe therapy can provide an
89 understanding of potential risk to the breastfeeding infant and will require confirmatory
90 studies.

1 INTRODUCTION

Breastfeeding is known to benefit maternal and infant health. Examples of benefits include lowered risks of postpartum depression, type 2 diabetes mellitus, breast cancer, and ovarian cancer in the mother; and reduced incidence and severity of respiratory tract infections and otitis media in the newborn and protection against allergic disease states in the infant [1-6]. Although the advantages to breastfeeding are clear, the decision to breastfeed becomes uncertain when there is maternal medication use. Contributors to this uncertainty are a lack of pharmacokinetic (PK) and pharmacodynamic (PD) information due to limited studies conducted in lactating mothers and their infants in the drug development process. Consequently, a review of drugs approved in the US between 2003-2012 found that nearly half of the labels (47.9%) had no data on breastfeeding, and only 4.7% presented human data [7].

Physiologically based pharmacokinetic (PBPK) modelling is a promising tool for improving our understanding of maternal medication dose to exposure relationships in breastfeeding infants. PBPK models use a mechanistic understanding of drug behaviour in virtual organisms in order to predict PK [8]. Thus, with knowledge about the drug properties combined with knowledge about the anatomy and physiology of the organism, drug exposures can be predicted. The main utility of PBPK modeling in maternal medication use during breastfeeding is the ability to extrapolate from adult to infants to predict levels of infant drug exposures which are difficult to directly observe. Given doses the infants would receive through breast milk, their exposures can be simulated.

Leveraging the utility of pediatric PBPK modeling, our group has developed the upper area under the curve ratio (UAR) [9]. The UAR is defined as the 95th percentile of simulated infant exposures to a maternal medication divided by a median therapeutic exposure of the

medication. Daily weight-normalized milk intake volumes are randomly selected from a non-linear model with variability derived from a literature review [10] and paired with a measured drug concentrations in milk to simulate doses in breastfed infants. These daily infant doses are then given to a population of virtual breastfeeding infants created from PBPK modeling. The 95th percentile of the resulting virtual breastfeeding infant drug exposures form the numerator of the UAR. Next, the drug PBPK model is used to simulate drug exposures in a population where therapeutic effects have been observed, with the median exposure informing the denominator. The UAR is proposed as an improvement over current metrics since it incorporates the anatomy and physiology of the infant, age-dependent factors (e.g., milk intake volume is a function of age), and variability in infant and maternal populations (e.g., maternal pharmacogenotypes resulting in varying drug in milk concentrations). Moreover, the UAR emphasizes exposure, whereas existing metrics such as the relative infant dose, are limited to dose information [11].

A medication that would offer further insight into use of the UAR is ezetimibe, an antihyperlipidemic agent that inhibits cholesterol absorption. Ezetimibe is the first line treatment for sitosterolemia, a rare genetic condition that often leads to increased atherosclerosis and premature vascular disease if left untreated. Women with this condition are often untreated for extended periods of time while child bearing due to a lack of safety data in pregnancy and breastfeeding [12]. Ezetimibe is classified as BCS II, which defines the drug with low solubility and high permeability [13]. Ezetimibe does not follow linear kinetics [14]. Absolute bioavailability has not been determined for ezetimibe since it is virtually insoluble in aqueous media, however, studies in dogs report a low bioavailability ($F = 0.58-1.1\%$) [15]. Ezetimibe and its primary active metabolite, phenolic ezetimibe-glucuronide (EZE-glucuronide) undergo extensive enterohepatic circulation as demonstrated through multiple animal studies [16, 17].

The multiple plasma concentration-time profile peaks can be observed in humans with oral administration [18]. Various factors contribute to ezetimibe and EZE-glucuronide clearance, including metabolism by UGT2B15 (~90% activity in human liver microsomes), UGT1A1, and UGT1A3 primarily in the liver. Additionally, several transporters are proposed to act on ezetimibe and EZE-glucuronide, including P-glycoprotein (MDR1), and OATP1B1, MRP2, and MRP3, respectively [19-21]. Both ezetimibe and EZE-glucuronide are highly protein bound (>90%) [22] and exhibit slow elimination (terminal half-life of 22 hours). Measured radiolabelled ezetimibe following oral administration in humans was excreted as 69% unchanged in feces at 96 hours [18]. The fraction excreted was likely a combination of unmodified ezetimibe and de-glucuronidated EZE-glucuronide entering the large intestine.

Scant information exists regarding maternal ezetimibe use during breastfeeding. Only animal studies have reported ezetimibe passage into breast milk [23]. Thus, the presence and concentration of ezetimibe in human breast milk are not known. Furthermore, there are no published pediatric PBPK models on ezetimibe and EZE-glucuronide. Maternal ezetimibe use is posited to be a concern during breastfeeding due to potential disruption of infant lipid metabolism [24]. However, this notion has not been formally studied in lactating mothers taking ezetimibe since sitosterolemia is a rare condition. Thus, the lack of interest in studying ezetimibe creates a severe therapeutic disadvantage for lactating mothers with sitosterolemia and their breastfeeding infants. Overall, information on ezetimibe and EZE-glucuronide concentrations in breast milk, infant exposure predictions, and applying the UAR workflow are urgently needed to better understand potential risk of ezetimibe.

The purpose of this work was to collect ezetimibe and EZE-glucuronide in breast milk samples from real world maternal use, develop and validate an assay to measure drug and

metabolite in breast milk concentrations, and perform the UAR workflow for the prediction of ezetimibe and EZE-glucuronide exposures in breastfed infants. Comparisons between predicted exposures in breastfeeding infants and therapeutic exposures would aid in our assessment of potential risk to the breastfeeding infant population.

2 METHODS

2.1 Workflow Overview

The overall workflow is presented as a schematic in **Figure S1**. A key component to the workflow was to create a population of virtual breastfeeding infants through a pediatric PBPK model. To build the pediatric PBPK model, an IV adult model was first developed using information on ezetimibe physicochemical and ADME properties and the anatomy and physiology of a mean adult. Next, ezetimibe oral parameters were defined for the mean adult and EZE-glucuronide was modelled. At each of these stages, uncertain parameters were optimized and the predicted plasma concentration-time profiles were evaluated against observed ezetimibe and EZE-glucuronide data. Once the IV and oral models were deemed appropriate, realistic variability on the anatomy and physiology of adults were applied to create a virtual adult population. The anatomy and physiology were then scaled to infants according to age, and relevant maturation factors were accounted for to produce a virtual breastfeeding population. The pediatric PBPK model was evaluated against children who were administered ezetimibe.

*To provide the virtual infants with ezetimibe doses through breastfeeding, an infant daily dose was attained by using an estimated volume of milk and ezetimibe and EZE-glucuronide concentrations as measured through voluntary breast milk samples. Briefly, a weight-normalized human milk intake (WHMI) volume model from Yeung [25] was used to select a volume from a normal distribution as a function of infant age (**Figure S1-A**), which was multiplied by a selected drug concentration in breast milk (**Figure S1-B**), resulting in an estimated infant daily dose*

(**Figure S1-C**). This process was repeated multiple times to create a distribution of infant daily doses (**Figure S1-C**) and served as the doses to provide virtual breastfeeding infants created by the aforementioned developed and evaluated ezetimibe PBPK model (**Figure S1-D**). The ezetimibe PBPK model was subsequently used to simulate infant exposures across age groups in comparison to adults (**Figure S1-E**). The methods of these steps are described in more detail in the sections below, from daily infant dose estimation, to pediatric PBPK model development and evaluation, and to predicted exposure simulations.

2.2 Study Population

Breast milk samples and study data (demographics, and breastfeeding- and sample-related information) were collected from two women recruited through the “Transfer of Ezetimibe into Breast Milk” study (Research Ethics Board (REB) #HS19991) at the University of Manitoba and the St. Boniface Hospital Lipid Clinic (PI: Dr. Pamela Katz), in collaboration with the project funded by the Canadian Institutes of Health Research (FRN 159782, PI: Shinya Ito). Inclusion criteria included women on chronic therapy with ezetimibe at steady state, lactating more than 1 week postpartum, and able to communicate in English.

2.3 Data Collection and Management

Breastfeeding mothers at steady state, taking a 10 mg daily dose of ezetimibe were asked to provide 10 mL of milk at two time points to collect hindmilk and foremilk. The hindmilk and foremilk samples were collected immediately before taking medication and up to four samples at any time after taking the medication, respectively. The milk samples were collected using an electric breast pump (a gift from Medela Canada Inc.). Milk samples were frozen at -80°C until analysis.

A questionnaire was used to collect: (1) Demographic data for the maternal-infant pair including date of birth, ethnicity, body measurements (body weight and height/length), pregnancy history (length, type of delivery (vaginal or cesarean section), pregnancy-related

complications, medical conditions, and medications during pregnancy), past medical history (any long-term illness or health condition and its treatment). (2) Additional information on the infant's general health condition, including symptoms requiring medical attention. (3) Specific information on breastfeeding type (exclusively breastfed, predominantly breastfed, or breastfed <80% of the infant's nutrition) and milk sample collection such as time and type of milk samples. Study data were collected and managed using REDCap electronic data capture tools hosted at the Hospital for Sick Children.

2.4 Modeling Software

PK-Sim version 11.1 (Open Systems Pharmacology Suite) was used as the PBPK modeling platform. Plot Digitizer version 2.6.8 (by Joseph Huwaldt) was used to digitize published PK and ontogeny profiles to obtain plasma concentration and enzyme activity across time data, respectively. R (R Core Team, 2019, Vienna, Austria) was used to model the ontogeny profiles, calculate measures of bias, and perform dose and exposure simulations. Ethics clearance for analysis of breast milk samples and study data was received through the University of Waterloo (REB #41155).

2.5 Analytical Methods

Ezetimibe and EZE-glucuronide concentrations in the breast milk samples were analyzed at the Pharmacology Research Unit, Sainte-Justine University Hospital Center.

2.5.1 Materials

Ezetimibe, ezetimibe phenoxy B-D glucuronide (EZE-glucuronide), ezetimibe-D4, and ezetimibe-D4 B-D glucuronide were purchased from Toronto Research Chemicals. Ezetimibe-D4 and ezetimibe-D4 B-D glucuronide are deuterated ezetimibe and EZE-glucuronide compounds, respectively, which were used to develop internal standards. Methanol, acetonitrile,

acetic acid, and ammonium hydroxide were purchased from Fisher Scientific. Ammonium acetate and formic acid were purchased from Sigma Aldrich. Water was purified by a Milli-Q water system. Donated breast milk samples from Hema-Québec were provided from multiple women to have a variety of matrices for the calibration curve and for quality control. All solvents and reagents were analytical or mass spectrometry grade. Qualitative and quantitative results were acquired by the Masshunter Acquisition and Masshunter Quantitative software (Agilent Technologies Inc.), respectively.

2.5.2 Assay Development

A high performance liquid chromatographic method was coupled with a tandem mass spectrometer to separate and quantify ezetimibe and EZE-glucuronide. Liquid chromatography-tandem mass spectrometry (LCMS/MS) Agilent Triple-Quad LCMS 6460c in negative mode with Eclipse XDB-C8, 4.6X150mm, 3.5 μ m column were used. The mobile phase consisted of 0.08% formic acid in water (40%, solvent A) and acetonitrile (60%, solvent B) for a total of 5-minutes sample run time at 0.8 mL/min in an isocratic mode. The expected transition ions were 408.1 $\rightarrow$ 271.1, 412.1 $\rightarrow$ 271.1, 584.1 $\rightarrow$ 271.1 and 588.1 $\rightarrow$ 271.1 for ezetimibe, ezetimibe-D4, ezetimibe phenoxy B-D glucuronide, and ezetimibe-D4 B-D glucuronide, respectively [26].

2.5.3 Assay Validation

Assay performance characteristics of selectivity, accuracy, precision, linearity and lower limit of quantification (LLOQ), matrix effect, carry-over, stability post-extraction, and recovery, were conducted using spiked breast milk controls from donated samples and standards. All performance studies were evaluated in accordance with Clinical and Laboratory Standards Institute guidelines. To be deemed acceptable, the performance metrics for linearity (standard concentrations), accuracy (mean accuracy, defined by % uncertainty = [mean differences

between measured concentrations and the assigned concentration) / (assigned concentration)] * 100%), and precision (coefficient of variation (%CV)) had to deviate less than $\pm 15\%$, and the LLOQ had to deviate less than $\pm 20\%$.

Selectivity of the assay was assessed in six breast milk samples from different blank samples of donated breast milk. Selectivity was ensured at the LLOQ for each compound and LLOQ was required to be larger than the background noise (signal-to-noise ratio of 5:1). Linearity and the LLOQ were evaluated by analyzing five calibration curves of eight (one curve per day) prepared standards by half-dilution and blank breast milk from a stock solution containing known amounts of certified ezetimibe and EZE-glucuronide (standards). The calibration curve was deemed reasonable if it consisted of six to eight points including the LLOQ (**Table S1**).

Accuracy was verified by analyzing three levels of controls (low, medium, and high) spiked from analyte powder and the LLOQ. Five samples were assessed per level per day. Accuracy was evaluated by calculating the percent deviation between the mean concentrations of the unknown samples, and true concentration of known samples. Over five days, within- and between-day precision was assessed by running five replicates of each quality-control level that included the LLOQ. The %CVs were calculated intra- and inter-day.

A matrix effect was assessed by comparing the analysis of ten different matrices spiked at a low concentration in duplicate. Carryover was checked by preparing one high (H) and one blank (B) sample. Each sample was dosed in triplicate (H1-3 and B1-3) and repeated five times. The percentage of carry-over was calculated according to Broughton [27]:

$$\text{Carry over (\%)} = \frac{B1-B3}{H3-B3} \times 100\% \quad (1)$$

Stability at post-extraction was verified with three levels of controls (low, medium, and high) that were spiked from powder and LLOQ at 4 hours and 24 hours. The concentration of each reinjection was compared with the initial injection to assess stability. Samples remained in the sampler that was either at a controlled temperature (10°C) or at room temperature. Percent extraction recovery was assessed by comparing the response of controls at three levels (low, medium, and high) and the LLOQ spiked in a breast milk matrix. The response consisted of four solutions of ezetimibe/EZE-glucuronide in the mobile phase (pure) which represented the amount of ng injected into the matrix of the three levels and the LLOQ.

2.5.4 Sample Preparation

An internal standard mixed working solution was created to correct for fluctuations present during the solid phase extraction (SPE; HLB Oasis SPE Cartridge, 3cc, 60 mg, Waters). The solution was created by pipetting 40 µL of ezetimibe-D4 at 5 µg/mL and 400 µL of ezetimibe-D4 B-D glucuronide at 10 µg/mL, mixed with 3,560 µL of acetonitrile. The resulting solution concentrations were 50 ng/mL ezetimibe-D4 and 1000 ng/mL of ezetimibe-D4 B-D glucuronide. In the first step, the sample was prepared. Human milk (blank, standard, quality control, or patient) was pipetted (400 µL) into a 1.5 mL conical tube. The internal standard mixing working solution was added (20 µL) and vortexed for 5 seconds. Afterwards, 600 µL of 0.2M ammonium acetate buffer (pH 6) was added. The samples were then vortexed for an additional 30 seconds and then set aside. The second step consisted of conditioning the SPE cartridge. The SPE cartridges were sequentially conditioned with 3 mL of methanol, 3 mL of nanopure water, and 3 mL of 0.2M ammonium acetate buffer (pH 6). In the third step, the sample in mixed in the buffer from the first step was passed through the SPE cartridge. The cartridges were washed with 1 mL of 5% methanol and 2% ammonia washing solution followed

by 3 mL of nanopure water. Methanol (1 mL) and then acetonitrile (1 mL) were used for elution. The eluate was evaporated with nitrogen steam and reconstituted with 100 μ L of mobile phase 40% A and 60% B. The column was injected with 20 μ L of the sample.

2.6 Dose Simulations

Random sampling was performed to select actual measured ezetimibe and EZE-glucuronide concentrations (ng/mL) from the breast milk samples. Daily weight-normalized volume of milk intake as a function of postnatal age (mL/kg) were sampled from a literature review-derived milk intake model as previously published [10]. Infant daily doses were simulated by multiplying an ezetimibe and EZE-glucuronide in milk concentration with a weight-normalized milk volume of intake that would be received by each virtual breastfed infant.

2.7 PBPK Model Development and Evaluation

2.7.1 Adult PBPK Model

A whole-body PBPK model was first established in adults for subsequent extrapolation to children and breastfed infants. Pediatric PBPK model development followed the workflow described by Maharaj, Barrett [28]. First, the disposition of ezetimibe was parameterized by data from dogs administered 5 mg/kg IV bolus [16]. In the dog study, plasma concentrations were 7,723 ng/mL at 5 minutes post-dose which then declined biphasically [16]. $C_{\max}$ provided a reasonable indication of volume of distribution and a logP was selected to match half-life (mean of 4.3 hours).

Next, a naïve oral model was set up for a mean male individual weighing 71 kg. Rodgers and Rowland [29-31] and Schmitt [32] prediction methods for partition coefficients were evaluated based on visual model performance for curve shape. Since the fraction excreted in urine from oral administration studies in humans was low (0.05-0.1% [19]), renal clearance was

negligible and glomerular filtration rate was set to 0. Clearance was partitioned as hepatic and biliary. To reduce the uncertainty in ezetimibe clearance mechanisms, the contribution by UGT2B15 was ascertained from Ghosal, Hapangama [33]. Although UGT1A1 and UGT1A3 are also proposed to contribute to ezetimibe clearance, UGT2B15 was shown to produce nearly 90% of EZE-glucuronide through *in vitro* inhibition studies [33]. The following equation was used to scale the intrinsic clearance of UGT2B15 in human microsomes (CL_{int}) to hepatic intrinsic clearance ($CL_{H,int}$) [34]:

$$CL_{H,int} = CL_{int}(UGT2B15) \times MPPGL \times liver\ weight \quad (2)$$

where MPPGL is the amount of microsomal protein per gram of liver. An MPPGL of 45 mg of protein/g of liver was used [35]. EZE-glucuronide served as the sole metabolite formed by UGT2B15 [33]. The remaining clearance of ezetimibe was attributed to MDR1 which transports compounds from the hepatocyte into bile [21]. MDR1 V_{max} was optimized using a Monte Carlo approach to explore the parameter space. All parameter optimizations were conducted with oral single dose datasets. The organ specific expressions of UGT2B15 and MDR1 were informed by reverse transcription polymerase chain reaction profiles (RT-PCR) in the PK-Sim expression database [36-38]. Reference concentrations (μM) of UGT2B15 and MDR1 were calculated from knowledge that on average, 30.5 pmol UGT2B15/mg protein [39] and 2.1 pmol MDR1/mg total membrane protein [40] are present.

Absorption parameters of the adult PBPK model consisted of a dissolution profile described by a Weibull distribution with dissolution half-time and shape indicative of a BCS Class II drug [41]. Specific intestinal solubility was manually optimized. To capture enterohepatic circulation and the emptying of the gallbladder, a 750 calorie meal was given at

study-specified times. Meal times were assumed based on visually inspecting the plasma concentration-time profile when the study did not provide information.

With the ezetimibe adult oral PBPK model established, EZE-glucuronide was modelled. The logP of EZE-glucuronide was modified by a factor of 1.37, which was the factor by which the ezetimibe *in silico* (ALOGPS) predicted value differed from the logP optimized in dog. The partition coefficient calculation algorithm for EZE-glucuronide followed that of ezetimibe. Clearance was described by MRP2 to transport EZE-glucuronide into bile [21]. MRP2 was optimized to the EZE-glucuronide plasma concentration-time profile. The expression and reference concentration of MRP2 was populated by the RT-PCR profiles and an average abundance of 0.3 pmol MRP2/million hepatocytes [42], respectively. Although *in vitro* studies have suggested OATP1B1 to transport EZE-glucuronide from the portal vein into hepatocytes, pharmacogenotype studies in humans appeared to show no significant differences in plasma concentration-time profiles between genotypes [20]. Therefore, OATP1B1 was not included in the adult PBPK model. Finally, EZE-glucuronide specific intestinal absorption was set magnitudes lower than ezetimibe to reflect the general properties of a glucuronide.

Studies reporting plasma concentration-time data with variability were used to develop an adult population model. Anatomy and physiology variability are captured in PK-Sim [43] while interindividual variability in enzyme concentration for UGT2B15, MDR1, and MRP2 were user-defined and described in the following section 2.7.2.

The optimized adult oral PBPK model was evaluated for predicting multiple dose and steady state kinetics using observed data. Model performance for bias and precision were calculated with average fold error (AFE) and absolute AFE (AAFE), respectively. Generally, an

AFE and AAFE of 0.7-1.3 and <2, respectively, for plasma concentration-time simulated versus observed data are indicative of almost no bias and good precision.

2.7.2 Pediatric PBPK Model

The adult oral PBPK model was scaled to a virtual infant population for simulating drug exposure through breast milk. Anatomy and physiology of adult were scaled to infants at different ages. Relevant growth and maturation processes, including metabolic capacity, glomerular filtration rate, protein binding, and body composition, were adjusted for [44, 45]. Variability was then applied to create the population of infants. The ontogeny profiles of UGT2B15, MDR1, and MRP2 were user-defined. UGT2B15 ontogeny was modeled using human liver microsomes protein content from 8 weeks gestation to 18 years (N = 236) [46] and glucuronidation activity from 0 to 69 years of postnatal age (N = 237) [47]. MDR1 and MRP2 ontogeny were modeled using protein content of 0 to 71 years of postnatal age (N = 110) [48]. Enzyme content or activity were normalized to adult activity and used to fit functions to describe the ontogeny profile. For all function fitting, an L1 metric, also known as the sum of absolute deviations, was used to estimate the geometric mean adult-normalized levels across postmenstrual ages (PMA).

To capture the rich UGT2B15 data at 0 to 1 years PMA reported by Divakaran, Hines [46], a polynomial function was used:

$$\text{Normalized Content or Activity} = B1 \times PMA + B2 \times PMA^2 + B3 \times PMA^3 + B4 \times PMA^4 + B5 \times PMA^5 \quad (3)$$

where B1-B5 represents coefficients determined through fitting the model. A virtual population (N = 6,000) was created across the PMA (0-1 years old) to assess variability in content or activity. Geometric standard deviations were applied on the determined geometric means of B1 -

B5 to capture the observed variability. Past 1 year PMA, content nor activity were age-dependent and thus 5,000 virtual individuals were created with a geometric mean of 1 and a uniform geometric standard deviation. For MDR1, a Hill function described by:

$$\text{Normalized Content} = \frac{kPMA^n}{PMA^n + A_{0.5}^n} \quad (4)$$

where k is the vertical transformation factor, n is the Hill coefficient, and $A_{0.5}$ is PMA at 50% activity. Variability in content was assessed with 6,000 virtual individuals across 0 to 71 years PMA. Following a geometric mean calculation of normalized content from the Hill function, a geometric standard deviation was applied to capture the observed variability. Finally, normalized MRP2 content was not age-dependent and thus a population size of 5,000 was created a geometric mean of 1 was used with a uniform geometric standard deviation.

Evaluation of the pediatric oral PBPK model was performed by simulating children 6-11 years old administered 10 mg tablet daily. The mean AUC_{0-24h} were compared between model-predicted and observed data from Kusters, Caceres [49].

2.8 Exposure Estimations: RID, Predicted $AUC_{0-\tau}$, and the UAR

To attain the relative infant dose (RID), the mean breast milk concentrations of ezetimibe was multiplied by the standard 150 mL/kg/day intake volume, which were then divided by a weight-normalized therapeutic dose of 10 mg daily in adult (70 kg).

To predict ezetimibe and EZE-glucuronide $AUC_{0-\tau}$ in plasma, infant populations of 100 individuals were simulated from the pediatric PBPK model for each age bin in days: >0 to 7, >7 to 14, >14 to 30, >30 to 60, and >60 to 365. The infant populations were based on the National Health and Nutrition Examination Survey (NHANES) population (50% female) [50, 51]. Due to the large uncertainty in ezetimibe absorption experienced in the adult models, administration to simulated breastfed infants was through the portal vein. The infants were each assigned 1 mg/kg

to attain a simulated $AUC_{0-\tau}$. Average concentration at steady state ($C_{avg,ss}$) over 24 h was calculated. Simulated infant daily dose from the selected weight-normalized milk intake volume and ezetimibe or EZE-glucuronide concentration in milk was then provided to each infant to attain $AUC_{0-\tau}$. Ezetimibe and EZE-glucuronide AUC_{∞} were converted into molar equivalents and summed together to represent the complete breastfed exposure to the active components of the medication. The UAR at each age group was then calculated by dividing the 95th percentile of $AUC_{0-\tau}$ in simulated breastfed infant by the median adult therapeutic $AUC_{0-\tau}$. Adult therapeutic $AUC_{0-\tau}$ were simulated from 100 women of 25-34 years of age using the NHANES population taking 10 mg of ezetimibe daily. For sensitivity analysis, the exposure simulation process described was repeated for the virtual infant population receiving ezetimibe as an oral solution.

3 RESULTS

3.1. Characteristics of the Maternal-Infant Pair, Breastfeed, and Breast Milk Samples

Two maternal-infant pairs were included in the study. Both mothers completed the study questionnaire and provided breast milk samples. A summary of their demographic and breastfeeding characteristics is provided in **Table 1**. The maternal-infant pairs contributed to a total of 15 breast milk samples (10 and 5 samples from Maternal-Infant Pair 1 and 2, respectively). One of the mothers reported starting their ezetimibe therapy approximately 4 years prior to sample collection. The time elapsed from the last ezetimibe dose to sample attainment, sample type (pre-feed, between feeds, or post-feeds), and ezetimibe and EZE-glucuronide measured concentrations from the developed and validated assay (section 3.2) are shown in **Table 2**.

3.2 LC-MS/MS Assay Validity

The chromatograms for the blank and representative chromatograms for ezetimibe and EZE-glucuronide in both the quality sample and breast milk sample of one of the patients are presented in **Figures S2-S4**.

Selectivity and LLOQ:LLOQ response for ezetimibe was 40 times higher than the response of breast milk without ezetimibe. LLOQ response for EZE-glucuronide was 250 times higher than response of breast milk with no EZE-glucuronide.

Based on the plotted expected versus observed concentrations, the assay method was linear over the range of 0.039-5 ng/mL for ezetimibe and 0.39-50 ng/mL for EZE-glucuronide ($R^2 = 0.99$, quadratic, 1/x weight).

Within- and between-day accuracy and precision using LLOQ and quality controls are presented in **Table 3** and **Table 4**. The table results show accuracy, intra-day precision, and between-day precision met the criteria for less than $\pm 15\%$ deviation. LLOQ also met the criteria of less than $\pm 20\%$ deviation.

Post-extraction stability at both room temperature and at 10°C exhibited no significant difference for both ezetimibe and EZE-glucuronide. Extraction recovery was between 65% and 91% for ezetimibe, and between 60% and 86% for EZE-glucuronide which included LLOQ and quality controls. No significant carry-over nor matrix effects were observed for ezetimibe and EZE-glucuronide.

3.3. Dose Simulations

Across all age groups, simulated weight-normalized milk intake volumes resulted in a normal distribution with mean of 120.5 mL/kg and standard deviation of 45.7 mL/kg. The simulated doses produced a lognormal distribution with a median of 60.1 ng/kg and interquartile range of 71.8 ng/kg.

3.4 PBPK Model and Evaluation

3.4.1 Adult PBPK Model

Table 5 presents the naïve and optimized ezetimibe and EZE-glucuronide models. **Table 6** shows the datasets used for development (single dose administration) and evaluation (multiple dose administration). Plasma concentration-time profiles of predicted versus observed for studies used in model optimization are presented in **Figure S5**. Ezetimibe and EZE-glucuronide model evaluation produced AFE of 0.98 and AAFE of 2.06, and AFE of 0.46 and AAFE of 3.26. Thus, the model for ezetimibe was reasonably non-biased and showed precision. Conversely, the EZE-glucuronide model performed sub-optimally in terms of bias and precision. However, a comparison of predicted and observed $AUC_{0-\tau}$ in **Table 7** suggest that the EZE-glucuronide performed adequately in exposure predictions. Comparisons of predicted and observed plasma concentrations over time for ezetimibe and EZE-glucuronide are presented in **Figure 1** and **Figure 2**, respectively. Results of the adult population models are shown in **Figure S5**.

3.4.2 Oral Pediatric PBPK Model

Results of population simulations to capture UGT2B15, MDR1, and MRP2 ontogeny variability are shown in **Figure S6**. Demographic information from the 6-11 year olds studied in Kusters, Caceres [49] is presented in **Table 8**. Model-predicted AUC_{0-24h} for ezetimibe and total ezetimibe (ezetimibe plus EZE-glucuronide) appeared to resemble observed values from Kusters, Caceres [49] (**Figure 3**).

3.5 RID, Predicted Exposures, and the UAR

The calculated RID for ezetimibe was 0.062%. Predicted AUC from 0-24 hours of simulated adults administered a therapeutic dose compared to virtual breastfed infants across the five age groups are depicted in **Figure 4**. The highest predicted AUC_{0-24h} of ezetimibe and EZE-

glucuronide in infants was 5.37 nmol·h/L from breastfeeding, which is approximately 11.6-fold less than the lowest predicted adult AUC_{0-24h} (62.42 nmol·h/L). Finally, the calculated UARs per age group are presented in **Table 9**. Results of the sensitivity analysis revealed that when administered as an oral solution, the 95th percentile AUC_{0-24h} (1.32 to 3.02 nmol·h/L) and UARs (0.0012 to 0.0027) across age groups were generally lower than direct administration into the portal vein.

4 DISCUSSION

This study details the first instance ezetimibe and EZE-glucuronide from maternal breast milk samples has been successfully measured and reported. Through the collection of voluntary samples where mothers demonstrated real world use, a LCMS/MS method of assay for the complex milk matrix was developed and validated. Moreover, the measured concentrations were used to perform the UAR workflow and predict ezetimibe and EZE-glucuronide exposures in breastfeeding infants. Attainment of the UAR demonstrated minimal overlap between infants at the highest risk of exposures and adults administered a therapeutic dose. This study demonstrated the first time the UAR was applied to both parent and active metabolite. The flexibility of the UAR to portray potential risk in multiple compounds is an improvement over the RID. For instance, the difficulty in ascertaining the maternal dose for the metabolite serves as a hinderance to calculating the RID. This adaptability of the UAR was also voiced as an advantage over current metrics from interviews with 28 healthcare providers advising breastfeeding mothers taking medications [52].

Despite this study reporting only a few samples, the information serves as an important addition to a sparse literature of drug concentrations in breast milk. Therefore, it is common for

case reports published on limited samples, for example a study by Saito, Kaneko [53] that reported the two milk samples from two women receiving oral pravastatin daily during pregnancy. Our present study takes these case studies one step further by providing information on predicted breastfed infant exposure.

The oral ezetimibe pediatric PBPK model was evaluated in children 6-11 years old administered 10 mg tablet daily. Both ezetimibe and total ezetimibe AUC_{0-24h} were relatively well-predicted by the model which gives confidence to our ability to predict exposures in infants. Notwithstanding, this study presents limitations to discuss. First, evaluating the model in children is less optimal than using infant data that can better resemble the breastfeeding population. Second, this study assumes 100% fraction absorbed with direct entry into the portal vein. Although a limitation, it was reasonable based on the difficulty of modeling ezetimibe adults and provided the most conservative approach to estimating potential breastfeeding infant exposures.

Third, there is a potential impact of storing the frozen breast milk samples at -80°C for several years. The stability of ezetimibe and EZE-glucuronide at this temperature for a prolonged period of time is currently unknown. Guo, Wang [26] have demonstrated the instability of ezetimibe and EZE-glucuronide in plasma. Additionally, other glucuronides present in breast milk that have been studied in more depth include lamotrigine-N2 glucuronide from the parent, lamotrigine. In studies with lamotrigine-N2 glucuronide in plasma, mixed results were found with regards to degradation in different temperatures [54, 55]. Overall, further studies are needed to assess this potential instability of ezetimibe and EZE-glucuronide in breast milk under our study conditions.

Fourth, the general underprediction of both ezetimibe and EZE-glucuronide models for adult plasma concentrations and AUC, and existing bias and imprecision of the EZE-glucuronide model presents a limitation. Essentially, the ezetimibe and EZE-glucuronide enterohepatic circulation process was non-identifiable. Several influx and efflux transporters are proposed to be involved between the portal vein and hepatocyte, hepatocyte and bile, and hepatocyte to systemic circulation. Moreover, transporters on the enterocyte may also play a role in ezetimibe and EZE-glucuronide clearance [33]. This work performed a large reduction in uncertainty in ezetimibe clearance by establishing UGT2B15 clearance from *in vitro* studies. However, the mechanism by which EZE-glucuronide is rapidly absorbed during the first few hours, and the repeated conversion of ezetimibe to glucuronide and vice versa were not captured, thus resulting in lower than expected plasma concentration predictions. This was particularly evident in the elimination phase where observed concentrations of both compounds showed slow elimination. In light of the developed model underpredictions, the possibility for the simulated ezetimibe and EZE-glucuronide exposures in breastfeeding infants could be higher and thereby increase the UAR. This has potential to be achieved with appropriately modeling the accumulation of ezetimibe and EZE-glucuronide observed with multiple dosing in adults (2-fold increase) [56].

Despite this study's limitations, we provide an early assessment with the first measured and published concentrations of ezetimibe in breast milk. Results of our work will require confirmatory studies, including conducting an FDA recommended "mother-infant pair study" as described in the Clinical Lactation Studies draft guidance [57]. A mother-infant pair study is the most involved study design that is performed when breast milk sample concentrations are known, and yet there are major uncertainties on the absorption of drugs in infants and their risk assessment. Since our work removes the necessity to acquire exploratory breastfed infant plasma

samples (e.g., $n = 50$) by leveraging pediatric PBPK, only a few samples are needed to confirm our results. Following these confirmatory studies, it would be of interest to reconsider the UAR for ezetimibe and EZE-glucuronide and its role in understanding the potential influence on breastfeeding infant exposures, especially for mothers with sitosterolemia requiring ezetimibe therapy.

5 CONCLUSION

This study is the first report of ezetimibe and EZE-glucuronide concentrations measured in breast milk. Although only a few samples are presented, knowledge about their low levels in breast milk is invaluable information to healthcare providers advising mothers who are breastfeeding and taking ezetimibe to treat the rare condition, sitosterolemia. Predicted ezetimibe and EZE-glucuronide exposures in breastfed infants were substantially lower than exposures in adults administered ezetimibe for therapeutic indications. As an early assessment of PBPK model-informed exposure predictions in breastfeeding infants, future studies should confirm these results.

564 **FIGURE LEGENDS**

565 **Figure 1.** Oral predicted versus observed mean ezetimibe plasma concentrations for evaluation
566 ($R^2 = 0.14$). The solid line represents the line of identity. qd: once a day. PO: taken by mouth.

567 **Figure 2.** Oral predicted versus observed mean EZE-glucuronide plasma concentrations for
568 evaluation ($R^2 = 0.016$). The solid line represents the line of identity. qd: once a day. PO: taken by
569 mouth.

570 **Figure 3.** Comparison of **(A)** ezetimibe and **(B)** total ezetimibe (ezetimibe + EZE-glucuronide)
571 AUC from 0-24 hours on day 14 of 10 mg daily administered to children 6-11 years old from
572 Kusters, Caceres [49] compared to pediatric PBPK-model simulated (“This Study”). The coloured
573 points represent AUC_{0-24} from each individual.

574 **Figure 4.** Simulated AUC_{0-24h} in molar equivalents of ezetimibe and EZE-glucuronide of adults
575 administered 10 mg daily compared to breastfed infants across five age groups.

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